1/PRTS

Description

A group of synthetic antimicrobial peptides

FIELD OF THE INVENTION

This invention relates generally to antimicrobial peptides, the methods for preparing these peptides and methods for inhibiting the growth of microbes such as bacteria, yeast and viruses utilizing the peptides of the invention. The peptides are also useful for inhibiting tumor cell.

BACKGROUND OF THE INVENTION

The recent research data have revealed that, besides their immune systems, almost all living organisms have an additional defense system against invasion of pathogenic microorganisms. They produce antimicrobial peptides in their bodies and use them as a defense means against pathogenic microorganisms. Antimicrobial peptides are composed of 20-60 amino acids, and their molecular weights are about 2000-7000D. Thus far, as many as about 200 antimicrobial peptides have been found from amphibians, insects, mammals, plants, microorganisms and fishes. The importance of peptides as physiologically active materials has been greatly recognized. These antimicrobial peptides are showing antimicrobial activity against a broad spectrum of microorganisms, including Gram-negative bacteria, Gram-positive bacteria, protozoa and fungi. Some of them are effective against both cancer cells and viruses. Most of the antimicrobial peptides kill target cells rapidly and specifically, and have unusually broad activity spectra.

By researching the primary structure and higher-order structure of the antimicrobial peptide, a lot of researcher find that when the antimicrobial peptide is in the hydrophobic environment of mimic membrane, its antimicrobial activity is related with the α-helix proportion. Another research result indicats that antimicrobial peptide kills the microorganisms by destructing the intact membrane of the microorganisms, which makes the membrane of the microorganisms leakage (Nakajima Y. et al., J. Biol. Chem, 262:1665-1669; Zasloff M. Nature, 2002, 415:389-395). So someone tries to search the antimicrobial peptides having stronger antimicrobial activity by increasing α-helix structure or heightening the positive charge amino acids proportion in the antimicrobial peptides (Broth W.B. et al., Antimicrobial Agents Chemotherapy, 2001, 45:1894-1895; Hong S.Y. et al., Peptides, 2001, 22:1669-1674).

DISCLOSURE OF THE INVENTION

The present invention provides a group of synthetic antimicrobial peptides. They were designed on the basis of analysis of the native antimicrobial peptides. The sequences of the peptides of the invention are provided as

follows:

Arg Phe Arg Leu Val Arg Arg Ile Val Leu Ala (A1-A2-A3-A4)(A1′-A2′-A3′-A4′)

Arg Phe Arg Leu Val Arg Arg Ile Val Leu Ala (A1-A2-A3-A4)(A1′-A2′-A3′-A4′)(A1"-A2"-A3"-A4")

Arg Phe Lys Leu Val Arg Arg Ile Val Leu Ala (A1-A2-A3-A4)(A1′-A2′-A3′-A4′)

Arg Phe Lys Leu Val Arg Arg Ile Val Leu Ala (A1-A2-A3-A4)(A1′-A2′-A3′-A4′)(A1"-A2"-A3"-A4")

Arg Phe Lys Leu Val Lys Arg Ile Val Leu Ala (A1-A2-A3-A4)(A1′-A2′-A3′-A4′)

Arg Phe Lys Leu Val Lys Arg Ile Val Leu Ala (A1-A2-A3-A4)(A1′-A2′-A3′-A4′)(A1"-A2"-A3"-A4")

Arg Phe Lys Leu Val Lys Lys Ile Val Leu Ala (A1-A2-A3-A4)(A1′-A2′-A3′-A4′)

Arg Phe Lys Leu Val Lys Lys Ile Val Leu Ala (A1-A2-A3-A4)(A1′-A2′-A3′-A4′)(A1"-A2"-A3"-A4")

Lys Phe Lys Leu Val Lys Lys Ile Val Leu Ala (A1-A2-A3-A4)(A1′-A2′-A3′-A4′)

Lys Phe Lys Leu Val Lys Lys Ile Val Leu Ala (A1-A2-A3-A4)(A1′-A2′-A3′-A4′)(A1"-A2"-A3"-A4")

Arg Phe Arg Leu Phe Arg Arg Ile Leu Val Gly (A1-A2-A3-A4)(A1′-A2′-A3′-A4′)

Arg Phe Arg Leu Phe Arg Arg Ile Leu Val Gly (A1-A2-A3-A4)(A1′-A2′-A3′-A4′)(A1"-A2"-A3"-A4")

Arg Phe Lys Leu Phe Arg Arg Ile Leu Val Gly (A1-A2-A3-A4)(A1′-A2′-A3′-A4′)

Arg Phe Lys Leu Phe Arg Arg Ile Leu Val Gly (A1-A2-A3-A4)(A1′-A2′-A3′-A4′)(A1"-A2"-A3"-A4")

Arg Phe Lys Leu Phe Lys Arg Ile Leu Val Gly (A1-A2-A3-A4)(A1′-A2′-A3′-A4′)

Arg Phe Lys Leu Phe Lys Arg Ile Leu Val Gly (A1-A2-A3-A4)(A1′-A2′-A3′-A4′)(A1"-A2"-A3"-A4")

Arg Phe Lys Leu Phe Lys Lys Ile Leu Val Gly (A1-A2-A3-A4)(A1′-A2′-A3′-A4′)

Arg Phe Lys Leu Phe Lys Lys Ile Leu Val Gly (A1-A2-A3-A4)(A1′-A2′-A3′-A4′)(A1"-A2"-A3"-A4")

Lys Phe Lys Leu Phe Lys Lys Ile Leu Val Gly (A1-A2-A3-A4)(A1′-A2′-A3′-A4′)

Lys Phe Lys Leu Phe Lys Lys Ile Leu Val Gly (A1-A2-A3-A4)(A1′-A2′-A3′-A4′)(A1"-A2"-A3"-A4")

Arg Phe Arg Gly Val Arg Arg Ile Leu Val Gly (A1-A2-A3-A4)(A1′-A2′-A3′-A4′)

Arg Phe Arg Gly Val Arg Arg Ile Leu Val Gly (A1-A2-A3-A4)(A1′-A2′-A3′-A4′)(A1"-A2"-A3"-A4")

Arg Phe Arg Gly Val Lys Arg Ile Leu Val Gly (A1-A2-A3-A4)(A1´-A2´-A3´-A4´)

Arg Phe Arg Gly Val Lys Arg Ile Leu Val Gly (A1-A2-A3-A4)(A1´-A2´-A3´-A4´)(A1"-A2"-A3"-A4")

Arg Phe Arg Gly Val Lys Lys Ile Leu Val Gly (A1-A2-A3-A4)(A1´-A2´-A3´-A4´)

Arg Phe Arg Gly Val Lys Lys Ile Leu Val Gly (A1-A2-A3-A4)(A1´-A2´-A3´-A4´)(A1"-A2"-A3"-A4")

Lys Phe Arg Gly Val Lys Lys Ile Leu Val Gly (A1-A2-A3-A4)(A1´-A2´-A3´-A4´)

Lys Phe Arg Gly Val Lys Lys Ile Leu Val Gly (A1-A2-A3-A4)(A1´-A2´-A3´-A4´)(A1"-A2"-A3"-A4")

Arg Trp Arg Ile Gly Arg Arg Ile Val Leu Ala (A1-A2-A3-A4)(A1´-A2´-A3´-A4´)

Arg Trp Arg Ile Gly Arg Arg Ile Val Leu Ala (A1-A2-A3-A4)(A1´-A2´-A3´-A4´)(A1"-A2"-A3"-A4")

Arg Trp Arg Ile Gly Lys Lys Ile Val Leu Ala (A1-A2-A3-A4)(A1´-A2´-A3´-A4´)

Arg Trp Arg Ile Gly Lys Lys Ile Val Leu Ala (A1-A2-A3-A4)(A1´-A2´-A3´-A4´)(A1"-A2"-A3"-A4")

Lys Trp Arg Ile Gly Lys Lys Ile Val Leu Ala (A1-A2-A3-A4)(A1´-A2´-A3´-A4´)

Lys Trp Arg Ile Gly Lys Lys Ile Val Leu Ala (A1-A2-A3-A4)(A1´-A2´-A3´-A4´)(A1"-A2"-A3"-A4")

Lys Trp Lys Ile Gly Lys Lys Ile Val Leu Ala (A1-A2-A3-A4)(A1´-A2´-A3´-A4´)

Lys Trp Lys Ile Gly Lys Lys Ile Val Leu Ala (A1-A2-A3-A4)(A1´-A2´-A3´-A4´)(A1"-A2"-A3"-A4")

Arg Trp Arg Leu Phe Arg Arg Ile Gly Ile Gly (A1-A2-A3-A4)(A1´-A2´-A3´-A4´)

Arg Trp Arg Leu Phe Arg Arg Ile Gly Ile Gly (A1-A2-A3-A4)(A1´-A2´-A3´-A4´)(A1"-A2"-A3"-A4")

Arg Trp Arg Leu Phe Lys Arg Ile Gly Ile Gly (A1-A2-A3-A4)(A1´-A2´-A3´-A4´)

Arg Trp Arg Leu Phe Lys Arg Ile Gly Ile Gly (A1-A2-A3-A4)(A1´-A2´-A3´-A4´)(A1"-A2"-A3"-A4")

Arg Trp Arg Leu Phe Lys Lys Ile Gly Ile Gly (A1-A2-A3-A4)(A1´-A2´-A3´-A4´)

Arg Trp Arg Leu Phe Lys Lys Ile Gly Ile Gly (A1-A2-A3-A4)(A1´-A2´-A3´-A4´)(A1"-A2"-A3"-A4")

Arg Trp Lys Leu Phe Lys Lys Ile Gly Ile Gly (A1-A2-A3-A4)(A1´-A2´-A3´-A4´)

Arg Trp Lys Leu Phe Lys Lys Ile Gly Ile Gly (A1-A2-A3-A4)(A1´-A2´-A3´-A4´)(A1"-A2"-A3"-A4")

Lys Trp Lys Leu Phe Lys Lys Ile Gly Ile Gly (A1-A2-A3-A4)(A1´-A2´-A3´-A4´)

Lys Trp Lys Leu Phe Lys Lys Ile Gly Ile Gly (A1-A2-A3-A4)(A1′-A2′-A3′-A4′)(A1"-A2"-A3"-A4")

Arg Phe Arg Val Ile Arg Arg Ile Leu Val Gly (A1-A2-A3-A4)(A1′-A2′-A3′-A4′)

Arg Phe Arg Val Ile Arg Arg Ile Leu Val Gly (A1-A2-A3-A4)(A1′-A2′-A3′-A4′)(A1"-A2"-A3"-A4")

Arg Phe Arg Val Ile Arg Lys Ile Leu Val Gly (A1-A2-A3-A4)(A1′-A2′-A3′-A4′)

Arg Phe Arg Val Ile Arg Lys Ile Leu Val Gly (A1-A2-A3-A4)(A1′-A2′-A3′-A4′)(A1"-A2"-A3"-A4")

Arg Phe Arg Val Ile Lys Lys Ile Leu Val Gly (A1-A2-A3-A4)(A1′-A2′-A3′-A4′)

Arg Phe Arg Val Ile Lys Lys Ile Leu Val Gly (A1-A2-A3-A4)(A1′-A2′-A3′-A4′)(A1"-A2"-A3"-A4")

Lys Phe Lys Val Ile Lys Lys Ile Leu Val Gly (A1-A2-A3-A4)(A1′-A2′-A3′-A4′)

Lys Phe Lys Val Ile Lys Lys Ile Leu Val Gly (A1-A2-A3-A4)(A1′-A2′-A3′-A4′)(A1"-A2"-A3"-A4")

(A1-A2-A3-A4)(A1′-A2′-A3′-A4′) or (A1-A2-A3-A4)(A1′-A2′-A3′-A4′)(A1"-A2"-A3"-A4") is the core structure of the antimicrobial peptides. A1, A1′or A1" is one selected from the group consisting of Lys and Arg. A2, A2′or A2" is one selected from the group consisting of Gly, Ala, Val, Leu, Ile and Phe. A3, A3′or A3" is one selected from the group consisting of Gly, Ala, Val, Leu, Ile and Phe. A4, A4′or A4" is one selected from the group consisting of Lys and Arg. The N-terminal end of the core structure (A1-A2-A3-A4) is linked with a sequence having 11 amino acids. The first or third or sixth or seventh amino acid of the sequence is one selected from the group consisting of Lys and Arg. The second amino acid of the sequence is one selected from the group consisting of Trp and Phe. The fourth or fifth or eighth or ninth or tenth or eleventh amino acid of the sequence is one selected from the group consisting of Leu, Ile, Ala, Val and Gly.

The synthetic peptides provided by the invention comprise their functional analogs derived from amino acid substitution, cyclization, replacement of L-amino acid with D-amino acid, deletion or addition.

One method for producing the peptides provided by the invention is solid-phase peptide synthesis. The other method for producing the peptide is expressing a nucleic acid sequence encoding the peptide in a host cell transformed with a recombinant vector. Then the peptide is expressed in the host cell. The vector is one selected from the group consisting of plasmid and virus. The host cell can be a prokaryotic cell, including Escherichia coli and Bacillus subtilis. The host cell also can be a eukaryotic cell, including yeast cell,

plant cell, insect cell and mammal cell. The peptide can be detected by mass-spectroscopy.

In order to research the relation between the structure and the function of the antimicrobial peptides provided by the invention, we used peptide synthesizer, which was purchased from ABI, to produce a group of peptides. Presented below are examples of the solid phase synthesis of these peptides. The sequences of GK-1, GK-2, GK-3 are provided as below.

GK-1:

Lys Trp Lys Leu Phe Lys Lys Ile Gly Ile Gly Arg Leu Leu Lys Arg Gly Leu Arg Lys Leu Leu Lys

GK-2:

Lys Trp Lys Leu Phe Lys Lys Ile Gly Ile Gly Arg Leu Leu Arg Arg Leu Leu Arg Arg Leu Leu Arg

GK-3:

Arg Trp Arg Leu Phe Lys Arg Ile Gly Ile Gly Arg Leu Leu Lys Arg Gly Leu Arg

To assay the MIC of three peptides GK-1, GK-2 and GK-3 of the present invention, 96-well microtiter plate was used (In Yup Park et al; FEBS Letters; 437(1998) 258-262), cecropin A1 and buforin II as control. The result indicated that the bactericidal activity of the peptides provided by the invention were stronger than the two native antimicrobial peptides.

Synthesis and detection the MIC of the functional analogs of the peptides were provided by the invention, which were deletion derivative and cyclization derivative. The result indicated that the bactericidal activity of the peptides provided by the invention were stronger than the two native antimicrobial peptides.

When the antimicrobial peptides kill the bacteria, they may act on the higher organism, include human cells. The reason is that the antimicrobial peptide kills the microorganisms by destructing the intact membrane of the microorganisms, which makes the leakage of the membrane of the microorganisms. So the hemolytic activity of the antimicrobial peptides is one of the standards of the toxicity of the antimicrobial peptides. If the peptides make the hematoglobin release, then the number of the OD_{490} can confirm the

intensity of the toxicity of the antimicrobial peptides. This experiment in the invention detected the hemolytic activity on the human red blood cells of the antimicrobial peptides. The results indicated that the hemolytic rate was very small, to confirm that the hemolytic activity of antimicrobial peptide was very low.

The results of acute toxicity test in Kunming mouse of the antimicrobial peptides indicated that the peptides had no toxicity. The experiment of the efficacy of antimicrobial peptide against the *Staphylococcus aureus* infection in mice demonstrated that antimicrobial peptide provided by the invention was effective against S. aureus infection in an acute infection model in mice.

The invention provides a group of new synthetic antimicrobial peptides. They can be produced by the method of solid-phase peptide synthesis. The other method for producing the peptide is expressing a nucleic acid sequence encoding the peptide in a host cell transformed with a recombinant vector. Then the peptide is expressed in the host cell. The antimicrobial peptides have broad spectral bactericidal activity on Gram-positive bacteria, Gram-negative bacteria, fungi. The bactericidal activity of the peptides are stronger than the native antimicrobial peptides. The peptides have no toxicty to animal and plant cells. The peptides protected completely from the *Staphylococcus aureus* infection in mice when administered at a dosage of 0.25 mg/kg, while Vancomycin, which was a specific medicine for *Staphylococcus aureus*, was 100% effective only at the dosage of 4.5 mg/kg. The results demonstrated that antimicrobial peptide provided by the invention was effective against S. aureus infection in an acute infection model in mice. The antimicrobial peptides provided by the invention can be used to produce the drug to treat the diseases induced by Gram-positive bacteria, Gram-negative bacteria, and fungi.

The peptides of the present can be used to prepare a drug for treating the pathologic microbes, fungus and/or virus infectious diseases.

The peptides of the present invention can also be used to prepare an antitumor drug.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG1 is mass-spectrogram for the antimicrobial peptide GK-2.

EXAMPLES

EXAMPLE 1

Preparation and purification of antimicrobial peptide

Prepare GK-1, GK-2 and GK-3. Prepare cecropin A1 and buforin II as control.

Sequence of cecropin A1 (see Morishima, I., etc, Comp. Biochem. Physiol., 1990, B 95 (3), 551-554):

Arg Trp Lys Leu Phe Lys Lys Ile Glu Lys Val Gly Arg Asn Val Arg Asp Gly Leu Ile Lys Ala Gly Pro Ala Ile Ala Val Ile Gly Gln Ala Lys Ser Leu

Sequence of buforin II (see Park, C.B., Biochem. Biophys. Res. Commun. 1996, 218 (1), 408-413):

Thr Arg Ser Ser Arg Ala Gly Leu Gln Phe Pro Val Gly Arg Val His Arg Leu Leu Arg Lys

Presented below are examples of the solid phase synthesis of these peptides. The peptide synthesizer was purchased from ABI, USA. After cleaving with high concentration TFA, the peptide was purified by reverse-phase column. The purified peptide was then analyzed by MS. The procedures in detail are provided as follows:

1. Preparation of antimicrobial peptide(GK-2, 0.1mmol)

All reagents are purchased from ABI, USA.

The sequence of peptide GK-2 is

N- Lys Trp Lys Leu Phe Lys Lys Ile Gly Ile Gly Arg Leu Leu Arg Arg Leu Leu Arg Arg Leu Leu Arg -C.

The Pioneer Peptide Synthesis System performs solid-phase synthesis, in which peptide chains are assembled on a solid support from the C-terminus, one amino acid at a time, elongating the chain toward the N-termius. Calculate the amount of support (Fmoc-Arg(Pbf)-PEG-PS, purchased from ABI, loading factor 0.19mmole/g) needed for the synthesis. Weigh the support and transfer it to the column. Removal of the Fmoc (9-fluroenyl-methyloxycarbonyl) protecting group from the terminal amine or the resin is accomplished by treating the resin with 20% solution of piperidine in N,N-Dimethylformamide (DMF).The required volume of the 20% solution of piperidine in DMF is calculated automatically on the scale of the reaction being run. The resin is then washed with DMF. The 9-fluroenyl-methyloxycarbonyl (Fmoc) protected amino acid was dissolved in

O-(7-Azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium exafluorophosphate (HATU)/diisopropylethylamine (DIPEA). The solution was recycled through the column for 30 minutes. The resin is then washed with DMF. Repeat the steps from removal to coupling till the end of synthesis (see *Pioneer Peptide Synthesiser User's manual* for details).

The resulting peptides were cleaved as follows:
After reaction, the resin was removed, to which was added B type cleavage cocktail (88% TFA, 5% phenol, 5% water, 2%TIPS), continue to reaction for about 2 hours at room temperature. Filtering, and to the filtrate was added 10-fold volume of pre-cold absolute ether. The precipitate was collected by centrifugation at 4000rpm for 10 minutes, and dried at room temperature.

2. Purification of antimicrobial peptide
Weigh an amount of dried peptide, resolved in 0.1% TFA. The peptide was purified by reverse-phase column (elution: 80% acetonitrile/0.1% TFA). Collect the elution fraction.

3. Identification of antimicrobial peptide
As shown in Fig.1, the molecular weight of antimicrobial peptide GK-2 is analyzed and calculated by MS:

(1) 734.8×4=2939.2, 2939.2-4=2935.2

(2) 979.1×3=2937.3, 2937.3-3=2934.3

(3) 1468×2=2936, 2936-2=2934

The calculated MW of GK-2 is 2934. The theory value calculated from the peptide sequence is 2932.74. The peptide prepared proved to be the designed GK-2 antimicrobial peptide. The certified antimicrobial peptide is stored for further use.

Antimicrobial peptide GK-1, GK-3 and natural antimicrobial peptides cecropin A1 and buforin II were prepared similarly to the preparation of GK-2 antimicrobial peptide.

Example 2
Expression of ***Antimicrobial Peptide*** GK-1 gene in E. coli

The bacterial expression vector pGEX-4T1 is used for bacterial expression in this example (Amersham Pharmcia Biotech). Antimicrobial peptide gene GK-1 was designed and synthesized and cloned into pGEX-4T1, then the expression vector containing GK-1 was transformed into E. coli JM109, GST-GK-1 fusion protein was expressed by IPTG inducing, GK-1 was obtained after cleavaging by thrombin.

ATP, IPTG, T4 polynucleotide kinase, T_4DNA ligase, Klenow Fragment, Restriction endonucleases are products of BIOLAB except for special indication. The agarose gel DNA extraction kit is product of shanghai sangon, primers for PCR amplification were synthesized by shanghai sangon. Thrombin cleavage kit from sigma.

With respect to the methods of DNA separation, purification, PCR reaction, enzyme cleavage, plasmid transformation, fragment collection, link reaction etc. are referred to Molecular Cloning: A Laboratory Manual (edited by Joe Sambrook, David Russell, Cold Spring Harbor Lab (CSHL) Press, 2001.). E.coli JM109 was cultured in LB liquid or solid medium.

We use E.coli bias coden design GK-1 gene sequence, the sequence as following: For cloning the mature protein, the 5' primer containing the BamHI (**GGATCC**) restriction site, The 3' primer containing the stop coden (**TAG**), the sequence contain 78bp.

The sequence of GK-1 gene was synthesized by DNA synthesizer. A DNA segment was amplified by PCR reaction. A pair of primers were P1: 5' **-CCTAGGTTTACCT**-3'和 P2: 3'-**CCGCCTGCTGAA**-5'. PCR reaction was as following: 94℃, 30 seconds; 45℃, 45 seconds; 72℃, 30seconds; 30cycles. The PCR product was cleaved by BamHI after it reacted with Klenow fragment. The fragment collected by agarose gel DNA extraction kit (procedure see the kit). The recycled fragment linked with pGEX-4T1 vector which was cleaved by BamHI and

SmaI, the recombinant plasmid transformed E.coli JM109, then transformants identified by SmaI. The fusion protein GST-GK-1 was induced to be expressed by IPTG. The fusion protein was purified by GST affinity column, and GK-1 antimicrobial peptide was obtained after it cleaved by thrombin. For the procedure, please see the kit.

GK-1 polypeptide sequence:

Lys Trp Lys Leu Phe Lys Lys Ile Gly Ile Gly Arg Leu Leu Lys Arg Gly Leu Arg Lys Leu Leu Lys

GK-1 gene sequence:

GGATCCAAATGGAAACTGTTTAAAAAAATTGGCATTGGCCGCCTGCTGAA ACGCGGCCTGCGCAAGCTGCTGAAATAG

Example 3

Expression of Antimicrobial Peptide GK-1 gene in yeast

ATP, IPTG, T4 polynucleotide kinase, T_4DNA ligase, Klenow Fragment, Restriction Endonucleases are products of BIOLAB except for special indication. The agarose gel DNA extraction kit is product of shanghai sangon, primers for PCR amplification were synthesized by shanghai sangon. Thrombin cleavage kit is available from sigma.

The DNA sequence of GK-1 gene which was cleaved by BamHI linked with the DNA sequence of GST, then the linked gene was cloned into pBluescriptSKII (from Stratagene company, USA). Recombinant plasmid was transformed into E. coli DH5α (from CMCC, Wuhan, P.R.C). The plasmid was identified by DNA sequencing. The plasmid was cleaved by EcoRI and XhoI, then linked to yeast expression vector pPIC9. pPIC9 is used for yeast expression in this example (from invitrogen). The expression vector containing GK-1 was then transformed into KM71 (from Invitrogen company, USA), GST-GK-1 fusion protein was induced to be expressed by methyl alcohol, and GK-1 was obtained after cleavage by thrombin.

With respect to the methods of DNA separation, purification, PCR reaction, enzyme cleavage, plasmid transformation, fragment collection, ligase reaction etc. are referred to Molecular Cloning: A Laboratory Manual (edited by Joe Sambrook, David Russell, Cold Spring Harbor Lab (CSHL) Press, 2001). KM71 was cultured in BMGY liquid or solid medium. When GST-GK-1 fusion protein was expressed, BMMY medium was used. BMMY medium supplied methyl alcohol to 1% every 24 hours.

We use yeast bias coden design GK-1 and GST gene sequence, the sequence as follows: For cloning the mature protein, the 5' primer containing the BamHI (**GGATCC**) restriction site, The 3' primer containing the stop coden (**TAG**) and EcoRI (**GAATCC**) restriction site, the sequence contain 84bp. Additional a XhoI (**CTCGAG**) restriction site at 5'-terminal of the GST was supplied.

Preparation of the sequence of GK-1 gene: the sequence of GK-1 gene was synthesized by DNA synthesizer. Amplify a DNA segment by PCR reaction. A pair of primer were P3: 5'CCTAGGTTTACCT3' and P4: 5'AAGTCGTCCGCC 3'. PCR reaction is performed as follows: 94℃, 30 seconds; 45℃, 45 seconds; 72℃, 30seconds; 35 cycles.

Preparation of the sequence of GST gene: designed a pair of primers, the sequences were as follows:

5' -CTCGAGATGTCCCCTATACTAGGTT-3';

5'- CAGTGCTACGCCGGCGAG-3'.

Amplify the GST gene segment of pGEX-4T1 vector by PCR reaction with P5 and P6. PCR reaction is performed as follows: 94℃, 30 seconds; 45℃, 45 seconds; 72℃, 30 seconds; 30cycles.

Link the PCR products to plasmid: GK-1 PCR products is cleaved by BamHI/EcoRI after reacting with Klenow fragment. The fragment was collected by agarose gel DNA extraction kit (see the kit manual for details). The recycled fragment was linked with pBluescriptSKII vector which was cleaved by XhoI/EcoRI and GST PCR products which were cleaved by BamHI/XhoI. E. coli $DH_{5\alpha}$ was transformed by the recombinant plasmid. The transformants were identified by antibiotic resistance test, restriction endonucleases etc, and then was identified further by DNA sequencing and cleaved by XhoI/EcoRI. The expression plasmid pPIC9-gst-gk1 was constructed by linking the plasmid to pPIC9 vector which was cleaved by XhoI/EcoRI. The recombinant plasmid transformed E. coli $DH_{5\alpha}$. Scan transformants by Ampicillin resistance test. Prepare the KM71 competent cell (Clare JJ, et al., Gene, 1991, 105:205-212). Then transported (1.5KV, 22.5uF) the recombinant plasmid pPIC9-gst-gk1 cleaved with SacI into KM71 cell. Spreaded the electroporated yeast onto YPD plate, scanned for the fusion protein positive clone after being cultured two days at 30℃.

The fusion protein GST-GK-1 was induced to be expressed by methanol in transformant. The fusion protein was purified by GST affinity column and obtained GK-1 antimicrobial peptide after cleavage with thrombin. See the kit manual for details.

GST-GK-1 fusion protein sequence:

Leu Glu Met Ser Pro Ile Leu Gly Tyr Trp Lys Ile Lys Gly Leu Val Gln Pro Thr Arg Leu Leu Leu Glu Tyr Leu Glu Glu Lys Tyr Glu Glu His Leu Tyr Glu Arg Asp Glu Gly Asp Lys Trp Arg Asn Lys Lys Phe Glu Leu Gly Leu Glu Phe Pro Asn Leu Pro Tyr Tyr Ile Asp Gly Asp Val Lys Leu The Gln Ser Met Ala Ile Ile Arg Tyr Ile Ala Asp Lys His Asn Met Leu Gly Gly Cys Pro Lys Glu Arg Ala Glu Ile Ser Met Leu Glu Gly Ala Val Leu Asp Ile Arg Tyr Gly Val Ser Arg Ile Ala Tyr Ser Lys Asp Phe Glu The Leu Lys Val Asp Phe Leu Ser Lys Leu Pro Glu Met Leu Lys Met Phe Glu Asp Arg Leu Cys His Lys The Tyr Leu Asn Gly Asp His Val The His Pro Asp Phe Met Leu Tyr Asp Ala Leu Asp Val Val Leu Tyr Met Asp Pro Met Cys Leu Asp Ala Phe Pro Lys Leu Val Cys Phe Lys Lys Arg

Ile Glu Ala Ile Pro Gln Ile Asp Lys Tyr Leu Lys Ser Ser Lys Tyr Ile Ala Trp Pro Leu

Gln Gly Trp Gln Ala The Phe Gly Gly Gly Asp His Pro Pro Lys Ser Asp Leu Val Pro Arg Gly Ser Lys Trp Lys Leu Phe Lys Lys Ile Gly Ile Gly Arg Leu Leu Lys Arg Gly Leu Arg Lys Leu Leu Lys

GST-GK-1 gene sequence:

TTAGAAATGTCTCCTATTTTAGGTTATTGGAAAATTAAAGGTTTAGTTCAACC
TACTCGTTTATTATTAGAATATTTAGAAGAAAAATATGAAGAACATTTATATGA
ACGTGATGAAGGTGATAAATGGCGTAATAAAAAATTTGAATTAGGTTTAGAAT
TTCCTAATTTACCTTATTATATTGATGGTGATGTTAAATTAACTCAATCTATGG
CTATTATTCGTTATATTGCTGATAAACATAATATGTTAGGTGGTTGTCCTAAA
GAACGTGCTGAAATTTCTATGTTAGAAGGTGCTGTTTTAGATATTCGTTATGG
TGTTTCTCGTATTGCTTATTCTAAAGATTTTGAAACTTTAAAAGTTGATTTTTT
ATCTAAATTACCTGAAATGTTAAAAATGTTTGAAGATCGTTTATGTCATAAAAC
TTATTTAAATGGTGATCATGTTACTCATCCTGATTTTATGTTATATGATGCTTT
AGATGTTGTTTTATATATGGATCCTATGTGTTTAGATGCTTTTCCTAAATTAGT
TTGTTTTAAAAAACGTATTGAAGCTATTCCTCAAATTGATAAATATTTAAAATC
TTCTAAATATATTGCTTGGCCTTTACAAGGTTGGCAAGCTACTTTTGGTGGTG
GTGATCATCCTCCTAAATCTGATTTAGTTCCTCGTGGTTCTAAATGGAAATTA
TTTAAAAAAATTGGTATTGGTCGTTTATTAAAACGTGGTTTACGTAAATTATTA
AAATGAGAATTT

Example 4

MIC Assay of several invention peptides

All strains used in the following examples were purchased from NICPBP.

To assay the MIC of three peptides GK-1, GK-2 and GK-3 of the present invention, 96-well microtiter plate was used, cecropin A1 and buforin II as control.

The minimum inhibitory concentrations (MIC) of the invention peptides were determined using methods described below:

The strain was recovered, inoculated into sloped medium, and grown overnight at 37℃. Typical clone selected were grown overnight at 37℃ in LB culture, diluted in the same medium to give concentrations of about 10^4-10^5 CFU/ml. The broth dilutions were set up in a 96-well microtiter plate by putting 100 μl of LB-S in every

well. Added diluted peptide to every well (10ul per well), cultured overnight at 37℃. The next day, the plates were scored for growth in the wells, and the MIC determined (In Yup Park et al.; FEBS Letters; 437(1998) 258-262). Results were summarized in table 1.

When the ratio of the growth concentration for the bacteria with antimicrobial peptides to that for the bacteria without antimicrobial peptides is greater than 90%, the concentration of antimicrobial peptides is the minimum inhibitory concentration (The minimum inhibitory concentration (MIC) is defined as the minimal concentration when the growth of bacteria is significantly inhibited).

Table 1. Compare of MIC against different bacteria of five antimicrobial peptides

	Strain	MIC of some antimicrobial peptides(ug/ml)				
		cecropin A1	buforin	GK-2		
				<-1		<-3
G+	Staphylococcus aureaus CMCC26003	16	4	0.4	0.2	0.5
	Bacillus subtilis DB430	12	6	4	4	5
	Bacillus pumilus CMCC63202	50	6	0.5	1	0.8
	Micrococcus lysoleikticus S1.634	50	8	1.0	0.8	1.2
	Micrococcus lutea CMCC28001	30	8	2	4	3
G-	Escherichia coli ATCC8099	20	16	1	0.5	1.6
	Klebsiella pneumoniaeCMCC46117	16	20	2	0.8	2
	B Subacute sclerosing panencephalitis CMCC50094	12	14	4	1	6
	Pseudomonas aeruginosa CMCC10104	18	20	10	12	1.8

Fungi	Candida albicans ATCC10231	50	30	8	10	11
	Saccharomyces cerevisiae ATCC9736	50	20	14	12	12

Lower MIC value means higher antimicrobial activity .

Example 5

MIC of functional analogs of invention peptides derived from cyclization, deletion

Design and synthesis functional analogs of invention peptides: GK-19 (deletion derivative) and GK-20 (cyclization derivative). Synthesis is performed on Pioneer Peptide Synthesiser. See *Pioneer Peptide Synthesiser User's manual* for details. After purification by reverse-phase column (see Example 1), the analogs were then subjected to MIC test (see Example 4). Results were summarized in Table 2.

Sequences of GK-19(deletion derivative) and GK-20(cyclization derivative):

GK-19:

Arg Phe Lys Leu Phe Lys Lys Ile Pro Arg Leu Leu Arg Arg Gly Leu Arg Lys Val Leu Lys

GK-20:

Lys Trp Lys Leu Phe Lys Lys Ile Gly Ile Gly Arg Leu Leu Lys Arg Gly Leu Arg Lys Leu Leu Lys

Table 2. **MIC of functional analogs of invention peptides** GK-19 and GK-20

Strain		MIC(ug/ml)	
		GK-19	GK-20
G+	Staphylococcus aureaus CMCC26003	1.0	0.8
	Bacillus subtilis DB430	2	2
	Bacillus pumilus CMCC63202	10	6
	Micrococcus lysoleikticus S1.634	4	2
	Micrococcus lutea CMCC28001	10	4
G-	Escherichia coli ATCC8099	2	8
	KlebsiellapneumoniaeCMCC46117	2	10

	B Subacute sclerosing panencephalitis CMCC50094	1	8
	Pseudomonas aeruginosa CMCC10104	4	10
Fungi	Candida albicans ATCC10231	16	18
	Saccharomyces cerevisiae ATCC9736	12	20

Lower MIC value means higher antimicrobial activity .

EXAMPLE 6

In-vitro hemolytic activity

This experiment was to detect the hemolytic activity of the antimicrobial peptides. The references were Cecropin A1 and buforinII, which were solid-phase peptide synthesized by our company. Blood sample was normal human blood.

The test step was shown below:

Human red blood cells was washed by PBS(PBS:35Mm phosphate buffer/0.15m NaCl, pH7.0). Suck 100ul 8% red blood cells suspension to 96-well plate, add 100ul antimicrobial peptide (including cecropin A1, buforinII, GK-1, GK-2, GK-3) to each well, then incubated at 37℃, after 1 hour, 1500rpm centrifuged for 5 minutes. Suck 100ul 4% red blood cells suspension to new 96-well plate, detect the hematoglobin releasing under 414nm by microplate reader. The negative control was PBS, the positive control was TritonX-100. The result was summarized in table 3:

Table 3. Results of hemolytic activity of five antimicrobial peptides

Concentration of antimicrobial peptide (ug/ml)	Hemolytic rate(%)				
	cecropin A1	buforinII	GK-1	GK-2	GK-3
12.5	0	0	0	0	0
25	0	0	0	0	0
50	0	0	0	0	0
100	1.2	0	0.5	0.2	0.6
200	3	0.5	0.8	1.0	1.1
500	10	1.7	1.5	2	1.9

The number of hemolytic rate in table 3 was smaller, the hemolytic activity of

antimicrobial peptide was lower.

Example 7

Acute toxicity test in Kunming mouse

The test was to detect the toxicity to animal of antimicrobial peptides including GK-1, GK-2, GK-3, provided by the invention. The references were Cecropin A1 and buforinII, which were solid-phase peptide synthesized by our company.

60 Kunming mouse, half was female, half was male, weight was 33.5±0.25g. The dosage of antimicrobial peptide was 1mg/kg, intramuscular injecting one time per day, consecutive 7days. We observed the reaction of the mouse under the maximum dosage. The result of the test demonstrated that the mice were normal and no abnormal reaction after 7 days intramuscular injection. It can be concluded that the antimicrobial peptides provided by the invention have no toxicity.

Example 8

Comparison of the efficacy of antimicrobial peptide and vancomycin against the *Staphylococcus aureus* infection in mice

The infection model was *Staphylococcus aureus* infection in the Kunming mouse. The test step was shown below:

S. aureus CMCC26003 was cultured overnight, with moderate agitation, in Veal Infusion broth (Difco) and diluted in broth containing 5% hog gastric mucin (Difco). Male Kunming mice weighing approximately 20 grams were infected intraperitoneally with 10^6-10^7 viable cells. There were 3 mice in each treatment group. Antimicrobial peptide GK-1 was administered intravenously (in 0.1 ml 5% dextrose for injection), within 10 minutes of infection. Vancomycin was administered subcutaneously.

Table 4. Efficacy of antimicrobial peptide and vancomycin against *Staphylococcus aureus* infection in mice

Dose (mg/kg)	Inhibitory rate (%)	
	GK-1	Vancomycin
	i.v.	s.c.
0	0	
0.125	20	
0.25	100	
0.5	100	0
1.0	100	40
2.0		80

4.0	100
8.0	100

As shown in Table 4, GK-1 protected 100% of the infected mice when administered at a dosage of 0.25 mg/kg intravenously. Vancomycin was 100% effective only at the dosage of 4.5 mg/kg. All of the untreated mice died in less than 24 hours.

This example demonstrated that antimicrobial peptide provided by the invention was effective against S. aureus infection in an acute infection model in mice using a highly virulent challenge dose of bacteria.

Example 9

Determination of the inhibitory activity against tumor cells

To determine the inhibitory activity of the peptides against tumor cells, a MTT colorimetric assay was performed. Fifty percent inhibitory concentrations of the antimicrobial peptides against tumor cells and normal fibroblasts were determined. Tumor cell K562 (human chronic myeloid leukemia cell), Bcap-37 (human breast cancer cell), QGY-7703 (human hepatocellular carcinoma cell), LOVO colon cancer cell, and mouse NIH-3T3 fibroblast were selected. These cells were provided by the College of Life Sciences, Fudan University. Cells were grown in RPMI-1640, containing 10% inactivated calf serum. Cells were transferred into the 96-well plate at 2*104 cells/well, and 150μl was added in each well. After the 96-well plate were incubated overnight at 37℃ in 5% carbon dioxide, 20μl diluted peptide solution was added to each well, then incubated for 3 days, 20μl MTT solution was added to each well, and incubated at 37℃ for 4 hours, 40μl, 002M HCl solution containing 20% SDS was added to each well to solve the purple crystal, and incubated overnight at 37℃. The absorbance at 570nm was determined.

Table 5. Result of the inhibitory activity against tumor cells (IC50)

	GK-1(ug/ml)	GK-2(ug/ml)	GK-3(ug/ml)
K562	45	56	13
Bcap-37	52	47	25
QGY-7703	38	44	21
LOVO	35	32	31
mouse NIH-3T3 fibroblast	>100	>100	>100

The results show that the antimicrobial peptides of GK-1, GK-2 and GK-3 all have antitumor activities, and the effect of GK-3 is the best.